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(71) Applicant: F. HOFFMANN-LA ROCHE AG [CH/CH]; Grenzacherstrasse 124, CH-4070 Basle (CH).

(72) Inventor: RENZETTI, Louis, Martin; 115 Elmwood Road, Verona, NJ 07044 (US).

(74) Agent: BRAUN, Axel; Grenzacherstrasse 124, CH-4070 Basle (CH).

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(57) Abstract

The present invention is directed to a method of combatting asthma with a composition containing an effective amount of a chimeric TNFα-binding protein which comprises the soluble part of the p55 TNF receptor and all domains except the first domain of the heavy chain constant region of a human IgG1 or IgG3 and to the use of such a chimeric TNFα-binding protein for the preparation of a medicament for the treatment of asthma.

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TREATMENT OF ASTHMA WITH TNFR-Ig

Asthma is a chronic inflammatory disease of the airways that is characterized by recurrent exacerbations due to exposure to specific allergens (IgE mediated response), exercise, cold air or stress. The hallmarks of inflammation associated with asthmatic disease are the presence of activated eosinophils, an increased sensitivity of the airways to nonspecific stimuli (airway hyperresponsiveness: AHR) edema, mucus hypersecretion and cough. This inflammatory process is believed to be mediated, in part, by the generation and activation of Th2-type lymphocytes which secrete a variety of cytokines. The cytokine TNFα is a cytokine which is involved in causing asthmatic conditions.

Multiple therapeutic agents are utilized for the treatment of asthma with inhaled β_2-agonists (relief of acute bronchospasm) and steroids (anti-inflammatory) being the agents of choice. No regimen is considered adequate, however, as the mortality of the disease is increasing. One of the most urgent unmet medical needs is for an agent that can rapidly reverse severe ongoing pulmonary inflammation as seen in acute/severe asthma. Further, none of the therapeutics may be classified as a disease modifying agent. There is certainly a need for a drug that can rapidly reverse the inflammatory response seen in acute/severe asthma as well as an agent that is a true disease modifier. The actions of TNFα promote asthmatic conditions, and applicants have determined that blocking the action of TNFα provides a means for relieving these conditions.

Applicants have now discovered a method for combatting asthma in patients suffering from an asthmatic condition comprising administering to said patient a composition containing a preparation composed of one or more chimeric TNF-α binding proteins, each of the proteins in said preparation being composed of the soluble portion of the p55 TNF receptor protein fused to an IgG wherein said fused IgG contains all of the IgG domains except for the first IgG domain of the IgG heavy chain constant region, said composition containing a therapeutically inert carrier, and said composition being

administered to said patient to provide the patient with an effective amount of said chimeric protein preparation to combat said asthmatic condition.

The composition may be administered effectively to a patient who is suffering an asthmatic attack where the chimeric protein preparations are administered in an amount sufficient to alleviate the effects of said attack. The composition may also be administered to an asthmatic patient prior to the onset of an asthmatic attack in an amount effective to prevent or retard the onset of the said attack.

This invention is directed to a method for combatting asthma in patients suffering from an asthmatic condition by administering to the patient a composition containing a preparation composed of one or more chimeric TNF-α binding proteins. The proteins in the preparation are composed of the soluble portion of the p55 TNF receptor protein fused to an IgG (immunoglobulin G) which contains all the domains except the first domain of the IgG heavy chain constant region (TNFR-Ig). The composition also contains a therapeutically inert carrier. The composition is administered to the patient so as to provide the patient with an effective amount of the chimeric protein preparation to combat said asthmatic condition.

The use of a chimeric TNF-α binding protein composed of the soluble portion of the p55 TNF receptor protein fused to an IgG wherein said fused IgG contains all of the IgG domains except for the first IgG domain of the IgG heavy chain constant region, for the preparation of a medicament for the treatment of asthma is a further object of the present invention.

The composition may be administered effectively to a patient who is in the course of an asthmatic attack, in an amount of protein preparation sufficient to alleviate the effects of the asthma attack. The composition may also be administered to an asthmatic patient prior to the onset of an asthmatic attack in an amount of protein preparation effective to prevent or retard the onset of the attack.

Any TNFR-Ig, i.e. chimeric TNF-α binding protein composed of the soluble portion of the p55 TNF receptor protein fused to an IgG wherein said fused IgG contains all of the IgG domains except for the first IgG domain of the IgG heavy chain constant region, may be used in a preparation of this invention to combat asthma in patients with an

asthmatic condition as described in the paragraph above. The IgG may be human IgG1 or IgG3, with IgG1 being preferred in this invention.

Examples of such TNFR-Igs include the proteins disclosed in EP 417 563, U.S. Patent Nos. 5,447,851 and 5,395,760, Lesslauer, et al. Eur.J. Immunol. 21(11):2883, 1991; Loetscher et al., J. Biol. Chem. 266(27):18324, 1991; Ashkenazi et al. PNAS (USA) 88:10535, 1991), which may be obtained by the methods also disclosed in these publications.

A preferred preparation of TNFR-Ig molecules of this invention is made with IgG1 and contains proteins which have a complex oligosaccharide terminated by one or more residues of sialic acid and have exposed N-acetylglucosamine, the molar ratio of sialic acid residues in the preparation being from about 4 to about 7 moles of sialic acid per mole of protein in particular about 5 to about 6, the molar ratio of exposed N-acetylglusosamine in the preparation being from about 1 to about 2 moles of N-acetylglucosamine per mole of protein, and the molar ratio of sialic acid residues to N-acetylglucosamine residues in the preparation being from about 0.35 to about 0.5 in particular about 0.4 to about 0.45. The preparation has an ioselectric point (pI) of from about 5.5 to about 7.5, which may be determined by chromatofocussing and which is sensitive to neuraminidase treatment.

TNFR-Ig of this invention may be obtained by conventional methods of recombinant technology or protein synthesis. DNA encoding the p55 TNF receptor, DNA encoding all domains except the first of an IgG1 or IgG3 heavy chain constant region, and ligating such sequences together for expression in an appropriate vector are known to a skilled person and described in the literature. Appropriate cloning vectors and host cells are well known and may be selected by a skilled person, and suitable culture conditions determined (see Animal Cell Culture: A Practical Approach 2nd Ed., Rickwood and Hames eds., Oxford University Press, NY 1992).

TNFR-Ig can then be purified using known methods of protein recovery and purification. The TNFR-Ig preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates. The culture medium or lysate may be centrifuged to remove particulate cell debris. The TNFR-Ig thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on immunoaffinity or ion–exchange columns; ethanol precipitation; reverse phase HPLC;

chromatography on silica or on a cation–exchange resin such as DEAE; chromatofocusing; SDS–PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G–75; and protein A Sepharose columns to remove contaminants such as IgG. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification. One skilled in the art will appreciate that purification methods suitable for the polypeptide of interest may require modification to account for changes in the character of the polypeptide upon expression in recombinant cell culture.

TNFR-Ig of this invention having specific sialic acid and N-acetylglucosamine ratios and pI may be obtained by using recombinant mammalian cells to express the TNFR-Ig and controlling the mammalian cell culture conditions under which the TNFR-Ig is produced as described, e.g. in WO 96/39488. Host cells selected should be capable of attaching N- and O-linked carbohydrates including sialic acid to the proteins they express. An example of such a host cell is a CHO cell. Suitable culture conditions are well known and depend on the selected host cell. Increasing cell specific productivity during the glycoprotein production phase results in a decrease in sialic acid content of the mature protein, and vice versa. Factors which affect cell specific productivity are well known in the art and include, but are not limited to, factors which affect DNA/RNA copy number, factors which affect RNA, such as factors which stabilize RNA, media nutrients and other supplements, the concentration of transcription enhancers, the osmolality of the culture environment, the temperature and pH of the cell culture, and the like. These factors may be adjusted by well known methods to obtain a preferred content of glycoprotein such as sialic acid. Varying the cell specific productivity of the production phase of the cell culture by adding an alkanoic acid such as sodium butyrate or a salt thereof to the cell culture at a concentration of about 0.1 mM to about 20 mM, or 5 to 20 mM, and engaging an osmolality of the cell culture at about between 250 and 600 mOsm, optionally in combination with one another during the transition phase produces a protein with differing amounts of sialic acid. A concentration of about 6.0 mM sodium butyrate and conditions of 350-400 mOsm provides a highly sialated TNFR-Ig of this invention, while a concentration of about 12 mM sodium butyrate provides a less sialated TNFR-Ig of this invention. The latter concentration may be combined with conditions of 450-550 mOsm.

The skilled practitioner will recognize that media osmolality is dependent upon the concentration of osmotically active particles in the culture fluid and that a number of variables that make up a complex mammalian cell culture medium impact osmolality. The

initial osmolality of the culture medium is determined by the composition of the culture medium. The osmolality can be measured using an osmometer such as that sold by Fisher Scientific, Pittsburgh, Pennsylvania, under the brand name OSMETTE (or the Osmette model 2007, available from Precision Systems, Inc. Natick MA), for example. In order to achieve an osmolality in the desired range, the concentration of various constituents in the culture medium can be adjusted. Solutes which can be added to the culture medium so as to increase the osmolality thereof include proteins, peptides, amino acids, hydrolyzed animal proteins such as peptones, non– metabolized polymers, vitamins, ions, salts, sugars (in particular glucose), metabolites, organic acids, lipids, and the like. In one embodiment, the osmolality is controlled by the addition of a peptone to the cell culture along with other components of the culture medium during a fed batch culture procedure.

Three phases of cell culture may be used, a growth phase during which cell growth in a selected mammalian host cell is maximized, followed by a transition phase in which cell culture parameters as described above for the desired sialic acid content of the mature glycoprotein are selected and applied, followed by a production phase of wherein parameters selected in the transition phase are maintained and glycoprotein product is produced and harvested. With regard to purification preferred within the context of the present invention are purification techniques and processes which select for the carbohydrates of the invention. The desired glycoforms of the present invention may be enriched for sialic acid–containing molecules by, for example, ion–exchange soft gel chromatography or HPLC using cation– or anion–exchange resins, wherein the more acidic fraction is collected.

Preferred TNFR-Igs of this invention have one or more of the following characteristics in purified composition: the range of pI of the TNFR-Ig composition is between about 5.5 and 7.5, the molar ratio of sialic acid to protein is about 4 to about 7 and especially about 5 to about 6, having about 1 to about 2 moles of exposed N–acetylglucosamine residues per mole of protein, having a molar ratio of sialic acid to N–acetylglucosamine of about 0.35 to about 0.5 and more preferably about 0.4 to about 0.45. The most suitable conditions for obtaining such a preferred TNFR-Ig employs culture conditions using sodium butyrate at a concentration of about 0.1 mM to about 6 mM, osmolality maintained between about 300-450 mOsm, and a temperature between about 30°C and 37°C.

Determination of the above characteristics of a TNFR-Ig may be made by a skilled person using well known techniques. For example, the complex carbohydrate portion of the glycoprotein produced by the processes of the present invention may be readily analyzed if desired, by conventional techniques of carbohydrate analysis. Thus, for example, techniques such as lectin blotting, well–known in the art, reveal proportions of terminal mannose or other sugars such as galactose. Termination of mono-, bi-, tri-, or tetra-antennary oligosaccharide by sialic acids can be confirmed by release of sugars from the protein using anhydrous hydrazine or enzymatic methods and fractionation of oligosaccharides by ion-exchange or size exclusion chromatography or other methods well–known in the art. The pI of the glycoprotein can also be measured, before and after treatment with neuraminidase to remove sialic acids. An increase in pI following neuraminidase treatment indicates the presence of sialic acids on the glycoprotein.

The carbohydrate structures of the present invention occur on the protein expressed as N–linked or O–linked carbohydrates. The N–linked and O–linked carbohydrates differ primarily in their core structures. N–linked glycosylation refers to the attachment of the carbohydrate moiety via GlcNAc to an asparagine residue in the peptide chain. The N–linked carbohydrates all contain a common Man1–6(Man1–3)Manß1– 4GlcNAcß1–4GlcNAcß–R core structure. Therefore, in the core structure described, R represents an asparagine residue of the produced protein. The peptide sequence of the protein produced will contain an asparagine–X–serine, asparagine–X– threonine, and asparagine–X–cysteine, wherein X is any amino acid except proline. O–linked carbohydrates, by contrast are characterized by a common core structure, which is the GalNAc attached to the hydroxyl group of a threonine or serine. Of the N–linked, and O–linked carbohydrates, the most important are the complex N– and O–linked carbohydrates. Such complex carbohydrates will contain several antenanary structures. The mono–, bi–, tri,–, and tetra–, outer structures are important for the addition of terminal sialic acids. Such outer chain structures provide for additional sites for the specific sugars and linkages that comprise the carbohydrates of the instant invention.

The resulting carbohydrates can be analyzed by any method known in the art including those methods described herein. Several methods are known in the art for glycosylation analysis and are useful in the context of the present invention. Such methods provide information regarding the identity and the composition of the oligosaccharide attached to the peptide. Methods for carbohydrate analysis useful in the present invention include but are not limited to lectin chromatography; HPAEC–PAD, which uses high pH

anion exchange chromatography to separate oligosaccharides based on charge; NMR; Mass spectrometry; HPLC; GPC; monosaccharide compositional analysis; sequential enzymatic digestion.

Additionally, methods for releasing oligosaccharides are known. These methods include 1)enzymatic, which is commonly performed using peptide–N–glycosidase F/endo–ß–galactosidase; 2) elimination using harsh alkaline environment to release mainly O–linked structures; and 3) chemical methods using anhydrous hydrazine to release both N– and O–linked oligosaccharides

Analysis can be performed using the following steps:

1. Dialysis of the sample against deionized water, to remove all buffer salts, followed by lyophilization.
2. Release of intact oligosaccharide chains with anhydrous hydrazine.
3. Treatment of the intact oligosaccharide chains with anhydrous methanolic HCl to liberate individual monosaccharides as O-methyl derivative.
4. N-acetylation of any primary amino groups.
5. Derivatization to give per-O-trimethylsilyl methyl glycosides.
6. Separation of these derivative, by capillary GLC (gas - liquid chromatography) on a CP-SIL8 column.
7. Identification of individual glycoside derivatives by retention time from the GLC and mass spectroscopy, compared to known standards.
8. Quantitation of individual derivatives by FID with an internal standard (13-O-methyl-D-glucose).

Neutral and amino–sugars can be determined by high performance anion–exchange chromatography combined with pulsed amperometric detection (HPAE–PAD Carbohydrate System, Dionex Corp.). For instance, sugars can be released by hydrolysis in 20% (v/v) trifluoroacetic acid at 100°C for 6 h. Hydrolysates are then dried by lyophilization or with a Speed–Vac (Savant Instruments). Residues are then dissolved in 1% sodium acetate trihydrate solution and analyzed on a HPLC–AS6 column as described by Anumula *et al.* (Anal. Biochem. 195:269–280 (1991).

Sialic acid can be determined separately by the direct colorimetric method of Yao *et al.* (Anal Biochem. 179:332–335 (1989)) in triplicate samples. In a preferred embodiment the thiobarbaturic acid (TBA) of Warren, L. J. Biol Chem 238:(8) (1959) is used.

Alternatively, immunoblot carbohydrate analysis may be performed. According to this procedure protein–bound carbohydrates are detected using a commercial glycan detection system (Boehringer) which is based on the oxidative immunoblot procedure described by Haselbeck and Hosel [Haselbeck *et al.* Glycoconjugate J., 7:63 (1990)]. The staining protocol recommended by the manufacturer is followed except that the protein is transferred to a polyvinylidene difluoride membrane instead of nitrocellulose membrane and the blocking buffers contained 5% bovine serum albumin in 10 mM tris buffer, pH 7.4 with 0.9% sodium chloride. Detection is made with anti– digoxigenin antibodies linked with an alkaline phosphate conjugate (Boehringer), 1:1000 dilution in tris buffered saline using the phosphatase substrates, 4–nitroblue tetrazolium chloride, 0.03% (w/v) and 5–bromo–4 chloro–3– indoyl–phosphate 0.03% (w/v) in 100 mM tris buffer, pH 9.5, containing 100 mM sodium chloride and 50 mM magnesium chloride. The protein bands containing carbohydrate are usually visualized in about 10 to 15 min.

The carbohydrate may also be analyzed by digestion with peptide–N–glycosidase F. According to this procedure the residue is suspended in 14 µl of a buffer containing 0.18% SDS, 18 mM beta–mercaptoethanol, 90 mM phosphate, 3.6 mM EDTA, at pH 8.6, and heated at 100 degrees C for 3 min. After cooling to room temperature, the sample is divided into two equal parts. One aliquot is not treated further and serves as a control. The second fraction is adjusted to about 1% NP–40 detergent followed by 0.2 units of peptide–N–glycosidase F (Boehringer). Both samples are warmed at 37° C for 2 hr and then analyzed by SDS–polyacrylamide gel electrophoresis.

TNFR-Ig of the present invention encompasses PEGylated TNFR-Ig, by which is meant a TNFR-Ig molecule which has been covalently conjugated to a polymer such as a polyalkylene glycol (substituted or unsubstituted) in particular a polyethylene glycol. Conjugation may be direct, but is preferably accomplished by means of various linking agents known in the art, for example those linkers disclosed in EP 510346, EP 593838, U.S. Pat. Nos. 4,766,106, 4,917,888, 4,902,502, 4,847,325, 4,179,337, 5,832,657, Veronese et al. Applied Biochem. and Biotech. 11:141 (1985), and Monfardini et al. Bioconjugate Chem. 6:62 (1995). PEGylated TNFR-Ig may be used in asthma treatment and prevention just as described for TNFR-Ig.

TNFR-Ig preparations of this invention are useful to combat asthma in asthmatic patients. In treating patients having an asthma attack, these preparations can alleviate the effects of the attack, such as reversing inflammation which has already occurred, and preventing further inflammation from occurring. When provided prophylactically, these preparations can delay or prevent onset of an asthma attack, or, an attack occurs, ensure that the attack is of decreased severity.

In accordance with this invention, the preparations can be administered for treatment or prophylactics to a patient in any conventional manner. Thus the preparation of this invention can be administered in conventional pharmaceutical compositions, which compositions include any conventional therapeutically inert carrier. The pharmaceutical compositions can contain inert as well as pharmacodynamically active additives. Liquid compositions can for example take the form of a sterile solution which is miscible with water. Furthermore, substances conventionally used as preserving, stabilizing, moisture-retaining, and emulsifying agents as well as substances such as salts for varying the osmotic pressure, substances for varying pH such as buffers, and other additives can also be present. If desired an antioxidant such as tocopherol, N-methyl-gamma-tocopheramine, butylated hydroxyanisole or butlyated hydroxytoluene can be included in the pharmaceutical compositions. Pharmaceutically acceptable excipients or carriers for compositions include saline, buffered saline, dextrose, or water. Compositions may also comprise specific stabilizing agents such as sugars, including mannose and mannitol, and local anesthetics for indictable compositions, including, for example, lidocaine. The previously mentioned carrier substances and diluents can be organic or inorganic substances, for example water, gelatine, lactose, starch, magnesium stearate, talc, gum arabic, polyalkylene glycol and the like. A prerequisite is that all adjuvants and substances used in the manufacture of the pharmaceutical compositions are nontoxic.

Preparations of this invention can be administered parenterally (for example by subcutaneous, intravenous, intramuscular, intraorbital, intracapsular, intraspinal, instrasternal types of injection) or by spray inhalation. Any conventional pharmaceutical preparation for parenteral or spray inhalation administration may be used. Examples of suitable pharmaceutical compositions are infusion or injection solutions. A preferred mode of administration is by intravenous injection. Another preferred mode of administration is by aerosol.

Any effective amount of the preparations of this invention to combat asthma may be used, to alleviate or reverse the effects of an attack or to prevent or retard onset of an attack. A preferred dosage of a TNFR-Ig composition for parenteral (injection) administration of this invention for treating, preventing, or reversing asthma provides from about 0.1 to about 5.0 mg of TNFR-Ig preparation per kg of body weight (mg/kg) per patient. A particularly preferred dosage is from about 1.0 to about 3.0 mg/kg. A preferred dosage of a TNFR-Ig composition for the same purpose and in a spray administration provides from 0.03% to 5.0% by weight of TNFR-Ig preparation. In either mode of administration, the treatment dosage may be administered once per day of treatment, or divided into smaller dosages and administered over about a 24 hour time period to reach a total given dosage.

For immediate treatment of an asthma attack by parenteral administration, a single dosage of 0.1 to 5.0, especially 1.0-3.0 mg/kg per patient per day may be given. For asthma prophylaxis by parenteral administration, such a dosage of 0.1 to 5.0, especially 1.0-3.0 mg/kg would preferably be provided at intervals from daily but preferably from twice weekly to once weekly to once monthly or another interval between, depending on the patient and conditions. Similarly for immediate treatment of an asthma attack by spray administration, a single dosage per day may be inhaled. For prophylaxis, such a dosage could be inhaled daily but preferably from twice weekly to once weekly to once monthly or another interval between, depending on the patient and conditions. The exact dosages in which the compositions are administered can vary according to the type of use, the mode of use, and the requirements of the patient, as determined by a skilled practitioner. The exact dosage for a patient may be specifically adapted by a skilled person in view of the severity of the condition, the specific formulation used, and other drugs which may be involved.

Spray compositions of this invention may be liquid or powder. They may be administered through the nose or through the mouth (nasally or orally). An example of a spray composition is an atomized spray which can be directed to the nose or mouth, produced from a spray atomizer by mechanical action on a water or saline solution of TNFR-Ig preparations. Another example is a nebulized spray, where a mist is produced from such a solution which is then actively inhaled by the patient (rather than being sprayed directly into the nose or mouth). If the TNFR-Ig is powdered, the particles should be large enough to be deposited in the respiratory tract rather than be exhaled by the patient after inhalation. Depending on the medical regimen for immediate treatment or prophylaxis, such a spray may be inhaled once or more daily, or less frequently as recommended for the patient. For spray administration, the patient is administered by inhalation an amount of a

TNFR-Ig of this invention effective to treat or prevent asthma as described above. The affected portions of the respiratory tract (nasal chambers, trachea, bronchi, lower air passages, or bronchioles) receive a suitable amount of TNFR-Ig by this means for asthma.

The spray formulations of this invention preferably provide from about 0.03% to 5.0% by weight of the preparation, more preferably about 0.03 to 0.5%, especially about 0.1 to 0.3%. A preferred spray formulation is an aerosol formulation. In general, the lower dosage ranges are preferred for spray such as nebulizer formulations and the higher dosage ranges are preferred for aerosols. Any conventional aerosol formulation may be used in this invention to provide an effective amount of the TNFR-IG preparation, preferably providing the amounts of preparation above. The preparation is most effectively administered by releasing a metered dosage of aerosol mist in the mouth of the patient while the patient inhales, taking the mist into the mouth and through the respiratory tract into the lungs. As described above, the effective amount may be administered orally or nasally at one time or in several smaller dosages throughout the day, and administered in a daily dosage for immediate treatment or at weekly to monthly intervals for prophylaxis.

A suitable aerosol formulation includes an appropriate dosage of the pharmaceutically active compound (e.g. TNFR-Ig preparation) and an effective amount of any conventional aerosol propellant. Any conventional surface active agent may also be included. The TNFR-Ig may be combined with the propellant as a liquid in solution or as a powder (for example as lyophilized by well-known methods). The propellant may be suitably liquified.

Those surface-active agents which are soluble or dispersible in the propellant are more effective. The more propellant-soluble surface-active agents are the most effective. It is also important that the surface-active agent should be non-irritating and non-toxic.

Any conventional propellant acceptable for use in a pharmaceutical composition known to a skilled person may be used, liquified or otherwise. The propellant may be one suitable for use with a powdered or one suitable for use with a liquid active ingredient, depending on whether the TNFR-Ig is in powdered or liquid form. The liquified propellant employed may be one which is a gas at room temperature (65° F.) and atmospheric pressure (760 mm of mercury), i.e., it may have a boiling point below 65° F. at atmospheric pressure and is non-toxic. Among the suitable liquified propellants which may be employed are the lower alkane containing up to five carbons, such as butane and

pentane. Examples of liquified propellants are the fluorinated and fluorochlorinated lower alkanes such as are sold under the trademark "Freon". Mixtures of the above mentioned propellants may suitably be employed.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Examples

Example 1: Plasmid encoding TNFR-Ig for use in production of TNFR-Ig

A. Cell Line

The Chinese hamster ovary (CHO)cell line used as the mammalian host cell line was derived from CHO–K1 (ATCC No. CCL61 CHO–K1). A CHO–K1 mutant dihydrofolate reductase ($DHFR^-$) deficient cell line named CHO–K1 DUX–B11 ($DHFR^-$) (obtained from Dr. L. Chasin of Columbia University; Simonsen, C.C., and Levinson, A.D., (1983) Proc. Natl. Acad. Sci. USA 80:2495– 2499; Urlaub G., and Chasin, L., (1980) Proc. Natl. Acad. Sci. USA 77:4216–4220) was then used to obtain a cell line with a reduced requirement for insulin by transfection with the vector containing the cDNA for the preproinsulin (Sures et al., (1980) Science, 208:57–59). The selected clone designated dp12.CHO requires glycine, hypoxanthine, and thymidine for growth, thus verifying their $DHFR^-$ genotype.

B. Construction of Soluble Type 1 TNFR–IgG_1 Chimera

A soluble type 1 TNFR–IgG_1 chimera was constructed by gene fusion of the extracellular domain of human type 1 TNFR with the hinge region and C_H2 and C_H3 domains of IgG_1 heavy chain (further referred to as TNFR1–IgG_1). Alternatively, the hinge region and CH2 and CH3 domains of the IgG3 heavy chain could also be used.

The human type 1 TNFR encoding DNA sequence (see Loetscher et al., supra) was obtained from the plasmid pRK– TNF–R [Schall et al., Cell 61, 361 (1990)]. To construct this starting plasmid, a 2.1kb placental cDNA clone (Schall et al., supra) was inserted into the mammalian expression vector pRK5, the construction of which is described in EP Pub. No. 307,247. This cDNA starts at nucleotide position 64 of the sequence reported by Loetscher et al., with the initiating methionine 118 bp downstream.

The source of the IgG_1 encoding sequence was the CD4–IgG expression plasmid $pRKCD4_2F_{c1}$ [Capon, D.J. et al., Nature 337, 525 (1989); Byrn et al., Nature 344, 667 (1990)], containing a cDNA sequence encoding a hybrid polypeptide consisting of residues 1–180 of the mature human CD4 protein (two N–terminal CD4 variable domains) fused to human IgG_1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region [Kabat et al., Sequences of Proteins of Immunological Interest 4th edition (1987)] which is the first residue of the IgG_1 hinge after the cysteine residue involved in heavy–light chain bonding), and ending with residue 441 to include the C_H2 and C_H3 Fc domains of IgG_1. See also EP 227110. Alternatively, the pCD4-Hγ3 vector (DSM 5523, EP 394,827) may be used. The CD4 cDNA is removed by SstI cleavage to obtain the desired IgG3 sequences.

TNFR1–IgG_1 was constructed by generating restriction fragments of plasmids pRK–TNF–R and $pRKCD4_2F_{c1}$ and ligating them, using deletional mutagenesis, so that threonine residue 171 of mature TNFR's is juxtaposed to aspartic acid residue 216 of IgG_1 heavy chain [Kabat et al., Supra]. The resulting plasmid pRKTNFR–IgG contained the full length coding sequence for $TNFR_1$ IgG_1. This plasmid may then be transfected into a host cell such as a CHO cell by conventional methods such as calcium phosphate precipitation. The resulting cells may be cultured by conventional methods to express TNFR-Ig, which may then be isolated by convention methods.

Example 2: Production of TNFR-Ig having about: 5-6 M sialic acid per protein, 0.4-0.45 M N-acetylglucosamine per protein, pI of 5.5-7.5

Cell Culture

The gene of Example 1 encoding the soluble type 1 TNFR–IgG_1 was introduced into dp12.CHO cells by transfection. This was accomplished using the calcium phosphate technique for introducing DNA into mammalian cells. Two days following transfection the cells were trypsinized and replated into selective medium (glycine–hypoxanthine and thymidine free Ham's F–12 DMEM, 1:1 v/v with 2% dialyzed serum). Subsequent isolates were screened for secretion of TNFR1–IgG_1. Clones expressing TNFR1–IgG_1 where amplified in methotrexate yielding high expressing clones and subsequently adapted to serum free medium. These cells were under a continuous selective pressure until transferred to nonselective medium for growth and expansion of the inoculum.

To provide cells for TNFR1–IgG_1 production cultures the cell population described above was expanded from the medium containing methotrexate by serial subcultivations in vessels of increasing volumes to growth medium containing no methotrexate. For these steps of the process the non selective growth medium was DMEM/HAM F–12 based formulation (see U.S. Patent 5,122,469, for example) with modified concentrations of some components, such as glucose, amino acids, salts, sugar, vitamins glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone (Primatone HS or Primatone RL), a cell protective agent such as Pluronic F68 (pluronic polyol) or the equivalent; Gentamycin; Lipid and trace elements.

The cultures were controlled at pH 7.2 +/– 0.4 by the use of CO_2 gas (acid) and /or Na_2CO_3 (base). Temperature was controlled near 37 °C during the growth period. Dissolved oxygen was maintained above 5% of air saturation by direct sparging with air and/or oxygen gas. The osmolality during the inoculum expansion phase was maintained between about 250 mOsm and 350 mOsm.

The growth phase for each culture was followed by a second phase or transition phase wherein culture parameters were changed from optimal growth to production conditions. During this transition phase the temperature of the culture system was decreased, generally to about between 30 and 35 °C. Butyrate was added and a certain osmolality range was engaged. Product accumulated during this production phase was analyzed for sialic acid content.

In a typical production schedule approximately 1.2×10^6 cells derived from the inoculum expansion from the selective stage where grown in a growth phase with a starting osmolality of 300 - 450 mOsm, preferably about 300. The growth medium was supplemented with trace elements, recombinant human insulin and hydrolyzed peptone. The cells were grown under these condition for 2 days. At the start of the third day the temperature of the cell culture was lowered. Simultaneous to or following the temperature shift, about 1 to 6 mmol sodium butyrate, preferably 6 mmol was added to the cell culture and the desired production osmolality was engaged by addition of various media components. Cells were grown under these conditions with feeding for 9–10 days. The cells were fed when necessary with various media components.

The pI of a heavily sialylated composition is lower than the pI of a lightly sialylated composition. Isoelectric focusing was performed for the composition. The isoelectric focusing gels separate the glycoproteins of the composition according to their isoelectric point, pI, using a pH gradient created with ampholytes of different pH. Analysis of the composition was performed using a pH gradient of 10 to 4.

The above composition exhibits an isoelectric point range, of about 5.5 to about 7.5 as determined by chromatofocusing, in which the pI is sensitive to neuraminidase treatment

Recovery of the TNFR–IgG

As described by Capon et al. Nature 337:525, 1989, IgG fusion proteins may be purified by centrifugation of cell culture and passage of resulting culture supernatant over a protein A column. Therefore, the cell culture obtained above was centrifuged and the supernatant applied to the protein A column. The TNFR1–Ig preparation was purified to greater than 95% homogeneity by this affinity chromatography on immobilized Staphylococcus aureus Protein A as described by Capon et al., supra.

Carbohydrate Analysis

A. Sialic acid content may be analyzed by the method of Warren, L. (1959) J. Biol. Chem. 234:1971–1975.

B. Release of intact neutral and amino–sugars may be determined by high pH anion–exchange chromatography combined with pulsed amperometric detection. Analysis was performed using the following steps:

1. Buffer exchange was performed with TNFR1–IgG_1 (approximately 50µg/ml) and the appropriate reference samples so that the final sample was contained in 1% acetic acid.
2. Approximately 90 µg TNFR1–IgG_1 as well as samples of reference materials were frozen in a dry ice and alcohol bath and the frozen sample lyophilized overnight.
3. The freeze dried samples were reconstituted in 500µl trifluoroacetic acid and incubated at 120° C for 1 hour.
4. After acid hydrolysis the TNFR1–IgG_1 and reference samples were cooled and evaporated to dryness.

5. Samples were reconstituted with water to a final concentration of approximately 0.6 mg/ml.
6. Separation of the monosaccharides was performed at ambient temperature by high pH anion exchange chromatography with pulsed amperometric detection using a Dionex CarboPac PA1 (4x250 mm) column (Dionex Corp., Sunnyvale, CA, USA).
7. Quantitation of individual monosaccharides was by comparison to reference monosaccharides.

Example 3. Preparation of TNFR- Ig

1. The CHO cell line DP12 (EP 307,247) was transfected with the plasmids pSV16B.TNFR-IgG (coding for TNFR1-IgG1) and pFD11 (coding for DHFR) using calcium phosphate co-precipation.

2. A cell clone derived from that transfection was scaled up into nine liter suspension cultures using selective medium containing 2% diafiltrated calf serum and 100 nmol/l methotrexate. Two nine liter cultures were inoculated into a 100 liter fermentor using serum-free medium without methotrexate. The 100 liter culture was inoculated into a 400 liter culture using serum-free meduim before the cells were inoculated into a 1000 liter production vessel after being washed 1000-fold with production medium to reduce residual serum components. The production medium was a DMEM/HAM F-12 - based formulation supplemented with glucose, amino acids, glycine, hypoxanthine, thymidine, recombinant human insulin, hydrolyzed peptone, pluronic F68, gentamycin, lipids, and trace elements.

3. The production culture was maintained for 13 days at a pH of 7.2 + or - 0.2 using CO_2 gas and/or Na_2CO_3. The cultivation temperature was changed from 37 C to 30 C after reaching a cell concentration of 5 x 10^6 cell per ml. The osmolality of the culture was adjusted to about 450 mOsmol/kg by adding media components on days 2 and 4. Sodium butyrate was added to a final concentration of 12 mM. The dissolved oxygen concentration was maintained at 60% air saturation by sparging the culture with air and/or oxygen.

4. The culture was harvested after 13 days and cells were separated from the supernatant by tangential flow filtration. This harvested cell culture fluid (HCCF) was concentrated approximately 20 fold using 10 KD Maxisette membranes. The retentate was

clarified and conditioned for Protein A chromatography by the addition of solid NaCl to 1.0 M followed by filtration through a 0.22 micron Durapore filter.

5. After the filtration the conditioned HCCF was passed over immobilized Protein A. The load material was followed by a wash several times prior to elution. The TNFR-IgG was step eluted with 0.05 M Na citrate/20% (w/v) glycerol, pH 3.0. The eluted pool was adjusted to pH 5.0 by the addition of 1 M Na citrate.

6. After the pH adjustment the Protein A pool was diluted and loaded onto an S-Sepharose Fast Flow. The load was followed by a wash then step elution with 0.05 M MOPS/0.05 M NaCl pH 7.2.

7. After the step elution the S-Sepharose Fast Flow pool was diluted then loaded onto a Q-Sepharose Fast Flow column. The column was eluted using a linear gradient from 0.0125 M NaCl to 0.125 M NaCl in 0.125 M MOPS, pH 7.2.

8. After the linear gradient elution the Q-Sepharose Fast Flow pool was conditioned by the addition of 1 volume of 0.1 M Na acetate/4.0 M urea/2.0 M ammonium sulfact, pH 5.0, then loaded onto a phenyl Toyopearl 650M column that had been equilibrated with 0.05 M Na acetate/2.0 M urea/1.0 M ammonium sulfate, pH 5.0. TNFR-IgG flowed through the column under these conditions. The load was followed by a wash with the equilibration buffer. The flowthrough and wash were combined to make the phenyl Toyopearl pool.

9. The two phenyl Toyopearl pools were combined and concentrated using 10KD Filtron Centrassette membranes. The retentate was passed over a G-25 column equilibrated in 0.01 M Na citrate/0.023 M glycine/0/023 M mannitol,. pH 6.0 to produce the TNFR-Ig composition.

The following Examples 4 - 10 describe in vivo experiments demonstrating that TNF-Ig of this invention alleviates effects of asthmatic attacks. The TNF receptor fusion protein TNFR-Ig markedly reduced, and in some instances abolished, responses evoked by antigen challenge in animal models of allergic lung inflammation. The magnitude of the inhibitory effect by TNFR-Ig was comparable to that obtained with the broad spectrum anti-inflammatory agent dexamethasone.

Abbreviations: TNFα, tumor necrosis factor-alpha; TNFR-Ig, recombinant, soluble TNF receptor IgG1 fusion protein; BAL, bronchoalveolar lavage; OA, ovalbumin; R_L, lung resistance; HBSS, Hanks balanced salt solution, substance P, a neuropeptide used to induce acute bronchospasm customarily referred to in the literature as substance P (see for example Selig and Tocker, Eur. J. Pharmacol. 213 (3):331-336 (1992).

Data Analyses. Mean +/- S.E.M. were calculated for all values in each experiment. Statistical differences were determined by a two-way repeated measures analysis of variance on ranked data followed by multiple comparisons testing using a Student Newman-Keuls t-test, or by Student's t-test. $P < 0.05$ was considered statistically significant. Calculations were performed using Microsoft EXCEL 5.0 (Softmart, Exton, PA, USA) and SigmaStat (Jandel Scientific, San Rafael, CA, USA) software packages run on a PC.

Drugs. All drugs were administered in a dosage volume of 1 ml/kg. TNFR-Ig was produced and purified as described in Example 3 and stock solutions prepared in a buffer of sodium citrate (10 mM), glycine (23 mM) and mannitol (230 mM), pH 6. Aliquots of stock solution were stored at -20 °C prior to use and dilutions were made in sterile saline. The following were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and prepared in sterile saline: ovalbumin, aluminum hydroxide, urethane, substance P acetate, (+/-) propranolol hydrochloride, dexamethasone 21-phosphate and Evans blue.

Example 4: Asthmatic symptoms in guinea pigs (airway hyperreactivity) alleviated with TNFR-Ig

This example uses guinea pigs rendered allergic to OA so that subsequent exposure to OA will induce the asthmatic symptom of airway hyperreactivity. This symptom is alleviated by TNFR-Ig, which is also compared to dexamethasone, a steroid known to alleviate asthma.

Male guinea-pigs were sensitized to OA (10 µg plus 1 mg $Al(OH)_3$ in 0.5 ml saline, s.c.) on day 0 and received the same dosage of OA as a booster on day 14. On day 21 the animals were challenged with a 0.1 % OA aerosol for 30 min. This sensitization causes asthma-like symptoms in the guinea pigs, including airway hyperreactivity to substance P. In order to determine the effects of TNFR-Ig on this symptom, TNFR-Ig was administered

before the challenge and the symptoms were compared with unchallenged animals and shown to be reduced. Comparable effects on airway hyperreactivity were obtained with dexamethasone (30 mg/kg, i.p., 1 hr before and 4 hr after challenge). Dexamethasone is a known treatment for asthma.

The guinea pigs were prepared for the experiments as follows, by being sensitized, challenged, and treated.

Sensitization. Male Hartley strain guinea pigs (Charles River, Kingston, NY, USA) weighing 250 to 300 g were actively sensitized to ovalbumin (OA) with a single s.c. injection (10 µg OA + 1 mg aluminum hydroxide in 0.5 ml sterile saline) on days 0 and 14 and used for study between days 21 and 28.

Challenge. The protocol for antigen challenge has been described previously (O'Donnell, et al., 1994). Sensitized animals were placed in a Plexiglas chamber and challenged with a 0.1 % (w/v sterile saline) OA aerosol for 30 min. The aerosol was delivered by a De Vilbiss Ultra-Neb 100 ultrasonic nebulizer (Breathing Services, Ephrata, PA, USA) using an airflow rate of 30 L/min that was driven by an internal fan. In order to minimize the number of deaths due to anaphylaxis, the nebulizer was cycled off and on for 60 sec during the first 5 min of exposure. Animal mortality was about 5 % under these conditions.

Drug Treatment. Groups of animals received either vehicle (see Drugs) or TNFR-Ig (1 or 3 mg/kg, i.p.) at 18 and 1 hr prior to OA aerosol. For experiments utilizing dexamethasone, separate groups of animals received either vehicle (saline) or dexamethasone (30 mg/kg, i.p.) at 1 hr before and 4 hr after OA aerosol. Dosing parameters for TNFR-Ig and dexamethasone were determined to be optimal on the basis of results from preliminary observations.

The results of this example show that guinea pigs sensitized to OA by exposure to OA, exhibited an enhanced airway reactivity to substance P (1-10 µg/kg, i.v.) at 6 hr after OA challenge. The enhanced reactivity to substance P is characteristic of asthma-like sensitization and shows that the guinea pigs were suffering from the asthma symptom of hyperreactivity induced by the above experimental conditions. The hyperreactivity was inhibited by TNFR-Ig (3 mg/kg, i.p., 18 and 1 hr prior to challenge).

The method used was as follows: Airway responses to substance P were assessed in both sensitized-unchallenged and -challenged guinea pigs 6 hr following OA aerosol exposure according to previously described methods (Selig and Tocker, 1992). The animals were anesthetized with urethane (2 g/kg, i.p.) and the carotid artery (blood pressure) and jugular vein (drug administration) were cannulated with PE-50 tubing. The trachea was cannulated with a 15-gauge needle and the animals placed in a whole body plethysmograph (Modular Instruments, Malvern, PA, USA). Spontaneous breathing was arrested with succinylcholine chloride (1.2 mg/kg, i.v.) and the animals were mechanically ventilated (Harvard Apparatus Model 683; South Natick, MA, USA) using a tidal volume of 1 ml/100 g body weight and a frequency of 60 breaths/min. The plethysmograph had a total volume of 2 L and the volume of the ventilatory circuit between the animal and the respirator was 10 ml. The plethysmograph was equipped with a Fleisch pneumotachograph (model #0000) that was connected to a Validyne differential pressure transducer (DP 45-14) for the measurement of airflow. Transpulmonary pressure was measured via a second Validyne transducer (DP 45-28) connected between a sidearm of the tracheal cannula and a 16-gauge intrapleural needle inserted between the fifth and sixth intercostal space. Airflow and transpulmonary pressure were recorded with a Modular Instruments (Malvern, PA, USA) M-3000 data acquisition system that was driven by an IBM 486DX2 PC. The computer utilized custom software (BioReport, Modular Instruments) for the calculation of lung resistance (R_L) based on the method of Amdur and Mead (1958). Readings were taken continuously and averaged over 10 sec intervals. Values of R_L were corrected for the internal resistance of the ventilatory circuit (0.11 cmH_2O/ml/sec). The animals received propranolol (1 mg/kg, i.v.) and were allowed to stabilize for 10 min before beginning administration of substance P.

Dosage-response effects of substance P (1, 3, 5 and 10 µg/kg, i.v.) were obtained by giving bolus injections at approximately 5 - 10 min intervals. Maximal changes in R_L were obtained for each dosage and expressed as a percentage of the baseline value determined before administration of substance P. ED_{200} values, defined as the dosage of substance P causing a 200 % increase in R_L, were determined for each animal by linear regression of log dosage-response curves.

Baseline values of R_L were about 0.30 cmH_2O/ml/sec and were not significantly ($P > 0.05$) different between groups. Sensitized, unchallenged guinea pigs were relatively insensitive to substance P, requiring a dosage of 30 µg/kg to increase R_L by at least 200 %

or greater (Table 1). In contrast, after OA challenge there was marked increase in the airway reactivity to substance P. ED_{200} values increased by about 10-fold (Table 1) with about a 5-fold increase in the maximum R_L obtained with the largest dosage of substance P (Table 1).

TABLE 1: Summary of the effects of TNFR-Ig and dexamethasone on the airway responses of the OA-sensitized guinea pig to substance P[a].

Treatment[b]	-log ED_{200}[c]	Maximum Response[d] (% Increase R_L)	n[e]
Unchallenged[f]	4.86 +/- 0.08	139 +/- 28	6
TNFR-Ig			
Vehicle	5.82 +/- 0.04	1811 +/- 228	7
1 mg/kg	5.87 +/- 0.01	1344 +/- 195	6
3 mg/kg	5.48 +/- 0.05*	714 +/- 69*	5
Dexamethasone			
Vehicle	5.87 +/- 0.05	935 +/- 91	5
30 mg/kg	5.35 +/- 0.04*	596 +/- 66*	6

[a]Dosage-response effects of substance P were determined at 6 hr after a 30 min challenge with a 0.1 % OA aerosol. Animals were pretreated with propranolol (1 mg/kg, i.v.) 10 min prior to examining airway responses to substance P.

[b]Animals were pretreated with the respective vehicles, TNFR-Ig (18 and 1 hr prior to OA challenge) or dexamethasone (1 hr before and 4 hr after OA challenge), i.p., using a dosage volume of 1 ml/kg.

[c]Values represent the negative logarithms (mean +/- S.E.M.) of the dosage (in g/kg) of substance P required to increase lung resistance (R_L) by 200 % of baseline.

[d]Values (mean +/- S.E.M.) represent the % increase in baseline R_L produced by substance P (10 µg/kg, i.v.).

[e]Number of animals per group.

[f]Dosage-response effects of substance P were examined in a group of sensitized animals that were not challenged with OA aerosol.

*Statistically significant ($P < 0.05$) difference in values between respective vehicle and drug treated groups.

Administration of TNFR-Ig (3 mg/kg) to sensitized animals significantly ($P < 0.05$) inhibited the OA-induced airway hyperreactivity to substance P. There was about a 3-fold decrease in the ED_{200} value for substance P and about a 60 % reduction in the maximum R_L (Table 1). A smaller dosage of TNFR-Ig (1 mg/kg) had no effect on the sensitivity to substance (Table 1). Treatment with dexamethasone (30 mg/kg) also significantly ($P < 0.05$) inhibited OA-induced airway hyperreactivity in the sensitized guinea pig by a similar degree to that obtained with the larger dosage of TNFR-Ig (Table 1).

Example 5: Asthmatic symptoms in guinea pigs (inflammatory cell influx) alleviated by TNFR-Ig

This example uses guinea pigs described in example 4 rendered allergic to OA so that subsequent exposure to OA will induce an asthmatic symptom, here inflammatory cell influx. This symptom is alleviated by TNFR-Ig, which is also compared to dexamethasone, a steroid known to alleviate asthma.

As in example 4, male guinea-pigs were sensitized to OA and challenged as described to induce asthmatic symptoms including airway inflammatory cell accumulation quantified at 6 24, 48 and 72 hr after challenge.. As in example 4, in order to determine the effects of TNFR-Ig on these symptoms, TNFR-Ig was administered before the challenge and the symptoms were compared with unchallenged animals and shown to be reduced. TNFR-Ig was also administered before challenge for inflammation, and shown to reverse inflammatory cell influx. Comparable effects on inflammatory cell accumulation at 6 and 24 hr were obtained with dexamethasone (30 mg/kg, i.p., 1 hr before and 4 hr after challenge).

The method used was as follows: Airway inflammatory cell influx was assessed at 6 and 24 hr post OA challenge by BAL according to previously described methods (Selig and Tocker, 1992). Briefly, guinea pigs were anesthetized with urethane (2 g/kg, i.p.) and tracheostomized with a 15-gauge catheter. Lungs were lavaged with 3 X 5 ml sterile Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco, Grand Island, NY, USA). The samples were centrifuged at 200g for 10 min at 25 °C and red blood cells were lysed from the resulting pellet with distilled water (1 ml for 30 sec) before restoring osmolarity by the addition of 10 ml of HBSS. Samples were centrifuged a second time (200g, 10 min, 25 °C) and the resulting pellet resuspended in 1 ml of HBSS. Total cell number was determined by Trypan Blue (Sigma Chemical, St. Louis, MO, USA)

exclusion from an aliquot of cell suspension using a hemocytometer. For differential cell counts, an aliquot of the cell suspension was centrifuged in a Cytospin (5 min, 1300 rpm; Shandon Southern Instruments, Sewickey, PA, USA) and the slides fixed and stained with a modified Wright's stain (Leukostat; Fisher Scientific, Pittsburgh, PA, USA). Standard morphological criteria were used in classifying at least 300 cells under light microscopy. The data were expressed as BAL cells X 10^6/animal.

Treatment with TNFR-Ig (1- 3 mg/kg, i.p., 18 and 1 hr and 1 hr pretreatments for BAL at 6 and 24 hr, respectively significantly ($P < 0.05$) inhibited the accumulation of neutrophils and total cells in BAL at 6 and 24 hr post OA.). TNFR-Ig (3 mg/kg, i.p.) also significantly ($P < 0.05$) reduced the number of eosinophils in BAL at both time points whereas a lower dosage (1 mg/kg, i.p.) had no effect. The results from the present study also show that the neutrophil component of the allergen-induced inflammatory response is mediated by TNF. Most notably, TNFR-Ig nearly abolished the influx of neutrophils in the BAL of sensitized guinea pigs 24 hr after antigen challenge with the number of neutrophils representing about 2 % of the total cells in BAL. Further, treatment with TNFR-Ig, but not dexamethasone produced a substantial reduction in neutrophils in the guinea pig at 6 hr after challenge. Blockade of TNF by TNFR-Ig or similar antagonists may cause an indirect reduction in factors known to contribute to the recruitment of neutrophils into the lung.

The cellular composition of the BAL of sensitized, unchallenged guinea pigs has been previously described (Selig and Tocker, 1992). Total cell counts in these animals averaged about 1 X 10^6/animal of which about 2 - 3 % are eosinophils and neutrophils. At 6 hr post OA, eosinophils and neutrophils comprised at least 40 and 20 %, respectively, of the total cell counts in animals treated with the vehicle for either TNFR-Ig or dexamethasone. The percentage of eosinophils in BAL remained constant at 24 hr, whereas the neutrophil counts declined to about 10 % of the total cell number. Inflammatory cell numbers and total cell counts were not different ($P > 0.05$) between the respective vehicle groups for TNFR-Ig and dexamethasone.

Inflammatory cell influx into the BAL of sensitized, challenged guinea pigs also was inhibited by dexamethasone (30 mg/kg). At 6 hr post OA, there was a significant ($P < 0.05$) reduction in the number of eosinophils and total cells in BAL which was similar to that caused by TNFR-Ig. There was no effect on the number of neutrophils at the 6 hr time point.0. At 24 hr post OA, the eosinophil, neutrophil and total cell counts in BAL were

significantly ($P < 0.05$) inhibited by dexamethasone . In comparison to TNFR-Ig, animals treated with dexamethasone had about 5-fold ($P < 0.05$) fewer eosinophils. In contrast, although dexamethasone treatment decreased the number of neutrophils in BAL, the proportion of these cells in the differential remained unchanged ($P > 0.05$) at about 8 % of total cell counts.

The ability of TNFR-Ig to reverse an ongoing inflammatory response was examined in sensitized guinea pigs. Animals were sensitized to OA, then challenged as described above. TNFR-Ig (3 mg/kg i.p.) was administered 30 min after OA challenge. Inflammation was determined by BAL at 24, 48 and 72 hr post challenge. For the 48 and 72 hr time points, TNFR-Ig was administered daily. Treatment with TNFR-Ig following challenge markedly reversed the influx of inflammatory cells in the BAL of sensitized guinea pigs.

Example 6: : Asthmatic symptoms in guinea pigs (lung edema) alleviated by TNFR-Ig

This example uses guinea pigs described in example 4 rendered allergic to OA so that subsequent exposure to OA will induce an asthmatic symptom, here lung edema. This symptom is alleviated by TNFR-Ig, which is also compared to dexamethasone, a steroid known to alleviate asthma.

As in example 4, male guinea-pigs were sensitized to OA and challenged as described to induce asthmatic symptoms including edema quantified at 6 hours after challenge.. As in example 4, in order to determine the effects of TNFR-Ig on these symptoms, TNFR-Ig was administered before the challenge and the symptoms were compared with unchallenged animals and shown to be reduced.

The method used was as follows: Airway microvascular leakage, a marker for lung edema, was quantified 6 hr post OA by the extravasation of Evans blue dye using methods described previously (Wasserman et al, Adv. Prostaglandin Thromboxane Leukotriene Res. 23:271-273, 1995). Guinea pigs were anesthetized with urethane (2 g/kg, i.p.) and the jugular vein catheterized. The animals then received Evans blue dye (30 mg/kg, i.v.) delivered over a 60 sec period. After 10 min the thorax was opened and a catheter (PE-240 tubing) was inserted through the left ventricle into the aorta. The ventricle was cross clamped, the right atrium cut and blood was expelled by perfusing the animal with 100 ml of saline using a cartridge pump (Masterflex; Cole-Parmer, Chicago, IL, USA) with a rate

of 100 ml/min. The pulmonary circulation was perfused with an additional 50 ml of saline by inserting the catheter in the pulmonary artery and cutting the left atrium. The lungs were removed en bloc and the trachea (distal 5 mm) and main bronchi were dissected away, blotted between filter paper and weighed. Evans blue dye was extracted in formamide (37 °C, 24 hr) and quantified by measuring the absorbance at 620 nm with a spectrophotometer (Beckman Instruments Model DU-64, Somerset, NJ, USA). Tissue dye content was interpolated from a standard curve of Evans blue dye concentrations (0.5 - 10 µg/ml) and expressed in ng/mg of tissue wet weight.

Using Evans blue dye as a marker of airway microvascular leakage, basal levels in the trachea and main bronchi from sensitized, unchallenged guinea pigs averaged 10 - 20 ng/mg of tissue wet weight. Six hours after OA challenge, Evans blue dye content in the trachea and main bronchus increased by about 5-fold. Treatment with either TNFR-Ig (1 or 3 mg/kg) or dexamethasone (30 mg/kg) attenuated ($P < 0.05$) OA-induced airway leakage at 6 hr post challenge in both the trachea and main bronchi.

TNFR-Ig (1 or 3 mg/kg, i.p.) abolished OA-induced airway edema (quantified by tissue content of Evans blue dye) in the trachea and main bronchi in sensitized guinea pigs.

Example 7: Asthmatic conditions in Brown Norway rats alleviated with TNFR-Ig

As in Example 4 with guinea pigs, inflammatory cell accumulation, an asthma symptom, was induced in rats and treated with TNFR-Ig. This model differs from the guinea pig in that allergic responses in the Brown-Norway rat are mediated by IgE.

The method used was as follows: Male Brown-Norway rats were sensitized to OA (1 mg OA plus 100 mg $Al(OH)_3$ in 0.5 ml saline, i.p.) on days 0, 1, and 2. On day 21, the animals were challenged with a 1 % OA aerosol for 30 min. Inflammatory cell accumulation was quantified by BAL at 24 hr after challenge. The protocol for sensitization, challenge and BAL was similar to that described above for the guinea pig with the exception of the following. Male Brown-Norway rats (Charles River, Kingston, NY) weighing 200 to 250 g were actively sensitized to OA with a single i.p. injection (1 mg OA + 100 mg aluminum hydroxide in 1 ml sterile saline) on days 1, 2, 3 and used for study on day 21 (Elwood et al., 1992). Separate groups of rats received either the

respective vehicle, TNFR-Ig (1 or 3 mg/kg, i.p.) or dexamethasone (0.3 mg/kg, i.p.) 1 hr prior to OA aerosol.

On the day of the experiment, rats were challenged with a 1 % OA aerosol for 30 min. BAL was performed at 24 hr post challenge by lavaging the lungs with 2 X 1 ml/100 g of HBSS. Red cells were lysed with 0.5 ml of distilled water and osmolarity restored with 5 ml of HBSS.

Treatment of the above rats with TNFR-Ig (3 mg/kg i.p., 1 hr prior to challenge) virtually abolished the accumulation of neutrophils in the BAL and caused a significant reduction in the number of eosinophils and total cell numbers. Similar results were obtained with dexamethasone (0.3 mg/kg, i.p., 1 hr prior to challenge). A lower dosage of TNFR-Ig (1 mg/kg, i.p.) also significantly reduced the number of neutrophils in the BAL, but had no effect on eosinophil and total cell numbers.

In more detail, sensitized, unchallenged Brown-Norway rats had a baseline total cell count in BAL of about 1 X 10^6/animal of which proportionally 1 - 2 % are eosinophils and neutrophils. Twenty-four hours after OA-challenge, the total number of cells in the BAL of the vehicle treated animals increased by about 3-fold with eosinophils and neutrophils representing at least 40 and 25 % of the cell population respectively. Inflammatory cell counts and the total number of cells in the BAL were not different ($P > 0.05$) between the respective vehicle treated groups.

Treatment with either TNFR-Ig (3 mg/kg, i.p.) or dexamethasone (0.3 mg/kg, i.p.) significantly ($P < 0.05$) reduced the accumulation of eosinophils, neutrophils and total cells in the BAL by similar degrees. Treatment with a lower dosage of TNFR-Ig (1 mg/kg, i.p.) significantly ($P < 0.05$) inhibited the number of neutrophils in BAL, but there was no effect on the accumulation of eosinophils or total cell counts in BAL.

Example 8: Reduction of neutrophilia in injured rat lungs

The rat model of Sephadex particle-induced lung inflammation exhibits an elevation in the number of eosinophils and neutrophils in the lung as well as granuloma formation that approximate those seen in chronic asthma. This model is used to show that TNFR-Ig reduces this chronic symptom.

The method used was as follow: Inflammatory cell accumulation into the lung was induced in male Sprague-Dawley rats by administering a suspension of Sephadex beads. (7.5 mg/kg i.v.). At 24 and 72 hr after Sephadex there was a significant increase in the total number of leukocytes in BAL fluid. At 24 hr, the number of neutrophils comprised around 50% of the total leukocyte number decreasing to around 10% of total by 72 hr. The eosinophil count was maintained at around 10% of the total leukocyte number.

Pretreatment with either TNFR-Ig (1 and 3 mg/kg, i.p., 1 hr prior to challenge) or dexamethasone (0.1 and 0.3 mg/kg, i.p.) inhibited the neutrophilia at 24 hr after Sephadex, although TNFR-Ig had no significant effect on total cell number. At 72 hr after Sephadex, TNFR-Ig (1 and 3 mg/kg, i.p., daily) significantly reduced the neutrophil influx into BAL fluid but had no inhibitory effect on eosinophil number. In contrast, dexamethasone (0.1 and 0.3 mg kg^{-1}, i.p., daily) virtually abolished the infiltration of neutrophils and eosinophils into BAL fluid. TNFR-Ig (1 and 3 mg/kg, i.p.) or dexamethasone (0.1 and 0.3 mg/kg, i.p.) significantly reduced total cell counts at the 72 hr time point .

Example 9: Attenuation of atopic asthma in primates

A primate model of atopic asthma employing wild caught cynomolgus monkeys that exhibit a natural airway sensitivity to *Ascaris suum* antigen was used to demonstrate that TNFR-Ig alleviates effects of the asthma symptom airway hyperreactivity. Repeated exposure to antigen induces asthma symptoms of airway in particular increased lung resistance (R_L). The monkeys when treated with TNFR-Ig showed reduction in R_L.

Wild caught naturally *Ascaris*-sensitive cynomolgus monkeys exhibit an enhanced airway responsiveness to inhaled methacholine and airway eosinophilia when subjected to repeated exposure to *A. suum* antigen. Thus the antigen was used to induce allergic reaction and consequent asthma symptom airway hyperreactivity in these monkeys. The following protocol was employed to examine the effect of TNFR-Ig on airway hyperreactivity.

Day 1: Determine dosage of methacholine (MCh) a bronchospasm-inducer like subtance P that produces a 100 % (PC_{100}) increase in lung resistance (R_L).

Day 3: Challenge monkeys with an inhaled dosage *Ascaris* antigen that produces at least a doubling in R_L.

Days 5 and 8: Repeat day 3.

Day 10: Repeat day 1. Determine the change in log PC_{100} values between days 1 and 10.

Administration of TNFR-Ig (3 mg/kg i.v.) on days 1, 3, 5 and 8 attenuated airway hyperreactivity to MCh.

Example 10: Allergic airway inflammation alleviated by TNFR-Ig in mice

A murine model of OA-induced allergic lung inflammation in mice was used to demonstrate that TNFR-Ig alleviates asthma symptoms of inflammatory cell influx. Sensitized mice that are repeatedly exposed to OA gradually develop airway hyperreactivity and an increase in eosinophil influx in the BAL. This model is associated with elevated levels of IgE and exhibits a classical Th2 cytokine profile (i.e. elevated levels of IL-4 and IL-5). TNFR-Ig was evaluated in this model for the ability to attenuate an on-going allergic inflammatory response.

The murine model of allergic lung inflammation is similar to the primate in that multiple exposures of sensitized animals to allergen is required to induce airway hyperreactivity and inflammatory cell influx. Female C57BL/6 mice were sensitized with OA (10 µg with 1 mg $Al(OH)_3$ gel, 0.1 ml, i.p.) on day 0 and then challenged daily from days 14-20 with a 1 % OA aerosol for 30 min. Airway hyperreactivity to MCh and BAL were performed 24 hr following the last OA challenge. Some lungs from sensitized, challenged were fixed for histological examination and others were homogenized for determination of TNF levels.

Inflammatory cell accumulation and TNF levels in the sensitized, challenged mouse peak following the final challenge (day 20) with OA aerosol. In the present study, TNFR-Ig (3 mg/kg i.p.) was administered immediately following the final exposure to OA. TNFR-Ig attenuated OA-induced hyperreactivity, but had no effect on inflammatory cell influx in the BAL . Daily administration of TNFR-Ig during the challenge period also was without effect on BAL cell counts. However, administration of TNFR-Ig (3 mg/kg, i.p., day 20) significantly reduced the number of eosinophils in the lung tissue of sensitized, challenged mice and abolished TNF levels in lung tissue.

Example 11: Aerosol compositions

The following are examples of aerosol compositions containing TNFR-Ig preparations of this invention

Example A - Aerosol

TNFR-Ig particle size range of 1 to 5 microns)	3.0%
Span® 85 (sorbitan trioleate)	1.0
Freon® 11 (trichloromonofluoromethane)	30.0
Freon® 114 (dichlorotetrafluoroethane)	41.0
Freon® 12 (dichlorodifluoromethane)	25.0

Example B - Aerosol

TNFR-Ig	0.5%
Span 85	0.5%
Propellant B[1]	99.0%

[1] Propellant B consists of 10% Freon 11, 50.4% Freon 114, 31.5% Freon 12, and 8.0% butane

Example C- Aerosol

TNFR-Ig	1.00%
Span 85	0.25%
Freon 11	5.0%
Freon W[1]	93.75%

[1] Freon W consists of 61.5% Freon 114 and 38.5% Freon 12.

Example D - Aerosol

TNFR-Ig	0.50%
Span 85	0.50%
Propellant (C)[1]	99.0%

[1] Propellant C consists of 30.0% Freon 11 and 70% Freon W.

Example E - Aerosol

TNFR-Ig	.88%
Sodium sulfate (anhydrous), micronized	.88%
Span 85	1.00%
Propellant consisting of 50% Freon 12, 25% Freon 11, and 25% Freon 114	97.24%

Example F - Aerosol	
TNFR-Ig	0.06%
Span 85	0.05%
Freon 11	20.0%
Freon 12/Freon 114 (20/80)	78.9%

Example 12: Injectable Composition

The following is an example of an injectable composition containing TNFR-Ig preparations of this invention.

Ingredient	Each ml contains
TNFR-IgG1	*
Citric acid anhydrous, USP	1.92 mg
Glycine, USP	1.70 mg
Mannitol, pyrogen-free, USP	41.90 mg
Sodium hydroxide	q.s. pH 6.0
Hydrochloric acid	q.s. pH 6.0
Water for injection, USP	q.s. to 1.0 ml**

* The concentration of TNFR-IgG1 used in this formulation may be 1.0 mg/ml to 20 mg/ml. The final amount of TNFR-IgG1 in this formulation is selected based on formulation concentration and volume per vial.

** Removed by lyophilization.

1 mg vial	(using 1 ml of a 1.0 mg/ml formulation)
2.5 mg vial	(using 1 ml of a 2.5 mg/ml formulation)
5.0 mg vial	(using 1 ml of a 5.0 mg/ml formulation)
10 mg vial	(using 2 ml of a 5.0 mg/ml formulation)
10 mg.vial	(using 1 ml of a 10.0 mg/ml formulation)
20 mg vial	(using 2.5 ml of a 8.0 mg/ml formulation)
20 mg vial	(using 4 ml of a 5.0 mg/ml formulation)
50 mg vial	(using 2.5 ml of a 20 mg/ml formulation)

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Claims:

1. A method for combatting asthma in patients suffering from an asthmatic condition comprising administering to said patient a composition containing a preparation composed of one or more chimeric TNF-α binding proteins, each of the proteins in said preparation being composed of the soluble portion of the p55 TNF receptor protein fused to an IgG wherein said fused IgG contains all of the IgG domains except for the first IgG domain of the IgG heavy chain constant region, said composition containing a therapeutically inert carrier, and said composition being administered to said patient to provide the patient with an effective amount of said chimeric protein preparation to combat said asthmatic condition.

2. The method of claim 1 wherein said patient is suffering an asthmatic attack and the chimeric protein preparations are administered in an amount sufficient to alleviate the effects of said attack.

3. The method of claim 1 wherein said composition is administered to an asthmatic patient prior to the onset of an asthmatic attack in an amount effective to prevent or retard the onset of the said attack.

4. The method of any one of claims 1 to 3 wherein the fused IgG is human IgG_1.

5. The method of any one of claims 1 to 4 wherein the proteins in said protein preparations have a complex oligosaccharide terminated by one or more residues of sialic acid and have exposed N-acetylglucosamine, the molar ratio of sialic acid residues in said preparation being from about 4 to about 7 moles of sialic acid per mole of protein, the molar ratio of exposed N-acetylglusosamine in said preparation being from about 1 to about 2 moles of N-acetylglucosamine per mole of protein, the molar ratio of sialic acid residues to N-acetylglucosamine residues in said preparation being from about 0.35 to about 0.5. and said preparation having an ioselectric point of from about 5.5 to about 7.5.

6. The method of claim 5 wherein the molar ratio of sialic acid to N-acetylglucosamine is from about 0.4 to about 0.45 and the molar ratio of sialic acid to protein is from about 5.0 to about 6.0.

7. The method of any one of claims 1 to 6 wherein said preparation is administered to said patients by an injection at a dosage of from 0.1 mg. to 5.0 mg. per kilogram per body weight of patient per day.

8. The method of claim 7 wherein the dosage is from1.0 mg. to 3.0 mg. per kilogram per body weight per day.

9. The method of any one of claims 1 to 8 wherein said preparation is administered by means of a mouth or nasal spray.

10. The method of claim 9 wherein said spray is a liquid spray composition containing from about 0.03% to 5.0% by weight of said preparation.

11. The use of a chimeric TNF-α binding protein composed of the soluble portion of the p55 TNF receptor protein fused to an IgG wherein said fused IgG contains all of the IgG domains except for the first IgG domain of the IgG heavy chain constant region, for the preparation of a medicamend for the treatment of asthma.